

The University of Chicago

SEROLOGICAL STUDIES OF
PLANT VIRUSES

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

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JORGEN M. BIRKELAND

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SEROLOGICAL STUDIES OF PLANT VIRUSES

J O R G E N M. B I R K E L A N D

Introduction

The discovery of viruses as supposedly different infectious agents has led to a problem of classification, the difficulty of which has become increasingly apparent.

Since the viruses could not be visually or culturally identified, they came to be characterized largely by what they did, that is, by the reactions they produced in the plant; and it became the custom to name the virus on the basis of the symptoms it produced on a single host (for example, tobacco mosaic, tomato mosaic, or cucumber mosaic). Confusion arose when later experiments showed: (1) that the symptoms produced by a given virus on a given plant vary with the source and mode of infection, the variety of the plant, its age, and the environment in which it has grown; (2) that the symptoms produced by a given virus may vary in different plants; and (3) that different viruses may produce similar symptoms on the same plant. Obviously a more reliable method of differentiation and classification of plant viruses is needed.

JOHNSON and HOGGAN (6) recommend that the present system be abandoned and an attempt be made to devise some system more comparable with the one used in classifying bacteria and fungi. Our knowledge, however, is as yet insufficient for classifying viruses, since present methods of study have yielded little positive information concerning their nature or characteristic properties which are of value in grouping them (10).

It has been shown that living things, plant and animal, by virtue of their chemical constituents have antigenic specificities which characterize the species, and that these antigenic specificities can be studied most advantageously by means of immunological or serological reactions. This approach has proved valuable in the study of the relationships, pathogenic properties, and chemical constituents of microorganisms (13), and might yield valuable information when applied to the classification of plant viruses.

The major difficulty in the use of these methods is that of securing a suitable preparation of the virus antigen. Since viruses cannot be grown in artificial mediums, it is difficult to secure antigens satisfactory in regard to purity and concentration. Such crude antigens as the extract of plant tissue contain antigenic substances other than the virus, which must be considered in interpreting the results. The few serological studies on plant viruses already made, however, although confined to the use of crude extracts and limited largely to the virus of tobacco mosaic, suggest that this method has possibilities.

Using the precipitin test, DVORAK (3) has shown that mosaic of potato affects the antigenic specificity of the globulin fraction of the cell juice and the cytoplasm. PURDY (8, 9) has shown that the juices from healthy plants and from plants affected with mosaic have antigenic substances in common. The juice of pathic tobacco plants contained an additional fraction which could not be removed from the homologous antiserum by absorption with healthy plant antigen. After such absorption, the anti-mosaic serum reacted with the juice (antigen) of tomato, petunia, pepper, and several species of tobacco, all of which had been infected with the virus of tobacco mosaic but not with the juice from healthy plants of these species. PURDY concludes from these results that the antigenic fraction which is present in the diseased plants and absent in healthy ones represents the virus, and not an altered host protein as was concluded by DVORAK. The work of MATSUMATO and SOMAWAZA (7) on tobacco mosaic substantiates PURDY's conclusions. These workers found that when the juice from tobacco and tomato plants affected with mosaic was digested with trypsin and used as a test antigen, reactions were not obtained with healthy plant antiserum but were obtained with mosaic plant antiserum. The tryptic digest was infectious, indicating that, although the healthy plant proteins were apparently broken down so far that they no longer acted as antigens, the virus had withstood the trypsin sufficiently to remain antigenic.

The characteristic antigenic property of a virus-diseased plant as reported by these workers might be due to (1) alteration by the virus of the normal healthy antigenic constituents of the plant, (2) linkage of the virus to the normal healthy constituents of the plant,

or (3) the virus itself. This paper deals with an attempt to determine which of the foregoing factors is responsible for the specificity of the serological reactions, and whether these reactions can be used in differentiating and classifying plant viruses.

Part I

PROBLEM

An attempt was made to determine whether the characteristic specificity of the serological reactions with plant virus is due to alteration of the normal healthy antigenic constituents of diseased plants or to the virus itself.

It was thought that purification of the virus by methods which involved different principles and testing of these preparations by the precipitin technique might produce evidence on the specificity of the reaction for the virus. In order to prepare serologically pure antigens it is necessary to remove contaminating antigenic substances. Since the preponderance of evidence indicates that only proteins or protein complexes can function as true antigens, it follows that the removal of plant proteins is of paramount importance in preparing serologically pure virus antigens. The test for purity of virus antigens is the presence or absence of a precipitin reaction with the healthy plant antiserum. If plant proteins are present in the virus antigen, the homologous antiserum will react with the juice of healthy plants used as antigen. If plant protein is not present, no precipitin reaction will be obtained. However, if the purified virus is antigenic and a precipitin reaction is obtained only with preparations containing the virus, the reaction can be logically attributed to its presence.

EXPERIMENTAL PROCEDURE

First, antigens were prepared from healthy tobacco plants and from mosaic (Tobacco Virus I Johnson) plants using several methods of purification; second, rabbits were immunized with these antigens; and, third, the immune sera produced were used for the precipitin ring tests. Readings of these tests were made after one and two hours' incubation at 25° C., and were confirmed after the tubes had remained in the icebox overnight.

PREPARATION OF ANTIGENS.—The five antigens used in this experiment were: healthy plant antigen,¹ crude mosaic antigen, acetone precipitate antigen, tryptic digest antigen, and tomato mosaic antigen $\text{Al}(\text{OH})_3$.

Young healthy tobacco plants were ground in a food chopper and the juice extracted from the resulting pulp by subjecting it to 5000 pounds' pressure. The juice was then centrifuged, and the liquid passed through a Berkefeld filter and used without further treatment as healthy plant antigen.

Juice from tobacco plants that had been inoculated with mosaic virus (Tobacco Virus I Johnson) was pressed out according to the method used in preparing healthy plant antigen, and divided into three portions which were treated individually to form three kinds of virus antigens. One portion was filtered through a Berkefeld V filter and used without further treatment as crude mosaic antigen. A second portion of 500 cc. of juice was treated with lead acetate and barium hydroxide to precipitate proteins, salts, and carbohydrates, then centrifuged, and the supernatant treated with two volumes of acetone. The acetone precipitate was filtered off and taken up in 50 cc. of distilled water. This suspension is hereafter referred to as acetone precipitate antigen. According to VINSON and PETRE (12) this method of purification gives high concentration of relatively pure virus. The third portion of the juice was digested for 48 hours with 2 per cent trypsin, passed through a Berkefeld V filter, and used as tryptic digest antigen. Coagulated egg white was added to one tube of the mosaic plant juice and trypsin to check the activity of the trypsin.

The method of BREWER, KRAYBILL, SAMSON, and GARDNER (2) was followed in preparing tomato mosaic antigen $\text{Al}(\text{OH})_3$. Tomato plants which had been inoculated with Virus I Johnson were ground in a food chopper, and the juice strained through gauze and super-centrifuged at 33,000 r.p.m. The residue was taken up in water, shaken vigorously, centrifuged, and the supernatant cleared with $\text{Al}(\text{OH})_3$. This suspension was highly infectious, as evidenced by the number of local lesions on *Nicotiana glutinosa* (4). It did not give a biuret test for protein.

¹ All plants were grown in the greenhouse.

Sodium chloride was not used to extract the ground plant tissue since there is a possibility that it might increase the plant proteins in the expressed juice.

PRODUCTION OF ANTISERA.—Rabbits were immunized in duplicate with the five antigens described. Intraperitoneal injections of 5 cc. each were given every third day. A test bleeding made after the fourth injection showed no reactions with the healthy plant antigen against its homologous serum. The rabbits were then given

TABLE I
PRECIPITIN TESTS WITH HEALTHY PLANT JUICES IN PRESENCE OF
HOMOLOGOUS AND HETEROLOGOUS ANTISERA

? = INDECISIVE, o = NO REACTION, + = FAINT RING, ++ = SMALL BUT DEFINITE RING, +++ = MODERATE RING, ++++ = WIDE RING

ANTISERUM	HEALTHY PLANT ANTIGEN DILUTIONS								CON- TROL NaCl
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	
Healthy tobacco..	+++	+++	+++	++	+	o	o	o	o
Crude mosaic....	+++	+++	+++	+++	++	+	o	o	o
Tryptic digest of mosaic plant...	?	?	?	o	o	o	o	o	o
Acetone precipitate of mosaic plant.	?	o	o	o	o	o	o	o	o
Tomato mosaic Al(OH) ₃	o	o	o	o	o	o	o	o	o
Control (normal rabbit).....	o	o	o	o	o	o	o	o	o

three more injections of 5 cc. each, and on the sixth day after the last injection bled aseptically. The blood was allowed to clot and the immune sera collected in sterile bottles.

PRECIPITIN RING TESTS.—The sera were pipetted into small precipitin tubes and layered with the desired dilution of antigen. The tubes were incubated at 25° C. for an hour and were then examined for the presence of a ring of precipitate at the interphase between the serum and the antigen. These readings were checked after another hour's incubation. The tubes were placed in the icebox overnight and examined for precipitate the next morning. All tests were run in duplicate. Each antigen was tested with homologous and heterologous antisera. Table I illustrates the method of setting up such a test with one antigen.

DISCUSSION

The results of preliminary experiments with crude extracts of healthy and diseased plants indicated that these materials had antigenic substances in common. (The precipitin tests are summarized in table II.) This is what is to be expected, of course, since unaltered plant protein was probably present in both types of preparation and would constitute a common antigen.

TABLE II
PRECIPITIN TESTS WITH FIVE ANTIGENS AND THEIR HOMOLOGOUS
AND HETEROLOGOUS ANTISERA
o = NO REACTION, + = DEFINITE PRECIPITATE

ANTISERUM (UNDILUTED)	ANTIGEN (DILUTION 1:4)						
	HEALTHY TOBACCO PLANT	CRUDE TOBACCO MOSAIC PLANT	ACETONE PRECIPI- TATE TO- BACCO MOSAIC VIRUS	AL(OH) ₃ PURIFIED TOMATO MOSAIC VIRUS	TRYPTIC DIGEST OF TOBACCO MOSAIC PLANT	CONTROL 2% TRYPSIN*	CONTROL NaCl
Healthy plant.....	+	+	o	o	o	o	o
Crude tobacco mosaic....	+	+	+	+	+	o	o
Acetone precipitate tobac- co mosaic virus.....	o	+	+	+	+	o	o
Al(OH) ₃ purified tomato mosaic virus.....	o	+	+	+	+	o	o
Tryptic digest of tobacco mosaic plant.....	o	+	+	+	+	+	o
Normal rabbit serum (con- trol).....	o	o	o	o	o	o	o

* Trypsin is antigenic and gives a precipitin test with tryptic digest antisera. However, this does not invalidate the results of the precipitin tests with other antigens.

Further experiments, summarized in table II, showed also that crude extracts of mosaic-diseased plants and purified preparations of virus from such extracts also had common antigenic factors. It might be expected that this would prove to be the case, but here the question arises as to whether the common antigenic substance or substances were (1) unmodified plant protein present in the crude extract and not entirely removed by the different methods of purification employed; (2) antigenically altered plant protein; or (3) the virus, *per se*, present in both crude and purified preparations.

Experiments were conducted in which antisera were prepared for virus material purified by the three methods already described. The precipitin tests, utilizing crude juice of healthy plants as an antigen in addition to the virus preparations, indicated that the purified virus preparations contained a common antigenic factor which was not present in the juice of healthy plants. This fact is of particular significance from two points of view. First, the methods of virus purification employed eliminate all or practically all unmodified plant proteins which may serve as antigens; second, the antigenic substance which gives rise to positive reactions with these antisera is not present in healthy plants.

The possibility that the common factor present in crude and purified preparations of virus is antigenically altered plant protein must be considered. However, inasmuch as virus purified by acetone precipitation and by tryptic digestion from tobacco as well as virus purified by $\text{Al}(\text{OH})_3$ adsorption from tomato were used as antigens, it seems improbable that the antigenic factor common to the purified preparations was antigenically altered plant protein.

FILTRATION EXPERIMENTS

During filtration experiments with Tobacco Virus I Johnson, it was noticed that the Seitz E. K. filter² removed virus, and that the filtrate was not infectious until several hundred cubic centimeters of juice had passed the filter. Since the virus was held back by the filter while most of the plant protein had passed through, it would seem that if the precipitin reaction is due to altered plant protein, the Seitz filtrate should give a precipitin test, unless the virus alters the protein so that it is not filterable. However, if the positive precipitin test is due to the virus, no reaction would be expected.

An experiment was conducted to test this hypothesis. Juice from mosaic tobacco plants was cleared by filtration through a Berkefeld V filter and divided into three portions, one of which was used without further treatment as crude virus I antigen. The second portion was filtered through a Seitz filter and used as antigen crude Seitz filtrate. The third was treated with $\text{Al}(\text{OH})_3$, according to the method of BREWER et al., to remove the plant proteins, and again divided

² The Seitz filters used were of 100 cc. capacity. Two pads were used.

into two portions. One part was filtered through a Berkefeld V filter and used as antigen $\text{Al}(\text{OH})_3$ Berkefeld filtrate; this filtrate was infectious. The other was passed through a Seitz filter and used as antigen $\text{Al}(\text{OH})_3$ Seitz filtrate; it was not infectious.

The antiserum used was obtained from a rabbit immunized with a purified preparation of tobacco mosaic virus from tomato.

The results given in table III tend to confirm the hypothesis that the precipitin reaction depends upon the presence of the virus, since

TABLE III
EFFECT OF SEITZ FILTRATION ON PRECIPITIN REACTION
OF SELECTED ANTIGENS
○=NO REACTION, +=DEFINITE PRECIPITATE

ANTISERUM (DILUTION 1:4)	ANTIGENS (DILUTION 1:2)				
	CRUDE MOSAIC	SEITZ FIL- TRATE OF CRUDE MOSAIC	TOBACCO MOSAIC $\text{Al}(\text{OH})_3$ BERKEFELD FILTRATE	TOBACCO MOSAIC $\text{Al}(\text{OH})_3$ SEITZ FILTRATE	HEALTHY PLANT
Crude mosaic virus I. . . .	+	+	+	○	+
$\text{Al}(\text{OH})_3$ tobacco mosaic. .	+	○	+	○	○
Healthy plant.	+	+	○	○	+

the purified virus antiserum reacts with crude or purified antigens which contain the virus but not with the Seitz filtrates of purified virus which do not contain the virus. Seitz filtrates of crude virus juice react with the healthy plant antiserum, indicating that the plant protein passes the filter.

RESULTS OF PRECIPITIN TESTS

1. Crude extracts of healthy and mosaic-diseased tobacco plants have antigenic substances in common.
2. Crude extracts of mosaic-diseased plants and purified preparations of the crude extracts have antigenic substances in common.
3. Antisera prepared for the virus purified by three different methods show a common factor which appears to consist of the virus, or a complex containing the virus, since the antisera reacted with

crude virus-containing plant juice but not with the crude juice of healthy plants.

4. Purified preparations of tobacco mosaic virus from tomato plants reacted with antisera for crude virus-containing juice from tobacco plants and with the antisera for purified preparations, but not with juice from healthy tobacco plants, indicating that the reactions secured were due to the virus and not to the plant protein.

5. Berkefeld filtrates of purified virus were infectious and reacted with the antisera prepared against virus-containing antigens.

6. Seitz filtrates of crude virus were not infectious and did not react with the antisera prepared for the purified virus, but did react with the crude mosaic antiserum and with healthy plant antiserum.

The results of PURDY and of MATSUMATO and SOMAWAZA on the virus of tobacco mosaic are thus confirmed. In addition, evidence has been obtained that the precipitin reactions secured are specific for the virus, or a complex containing the virus, since similar results are obtained regardless of the method employed in purification of the virus.

Part II

PROBLEM

An attempt was made to differentiate several plant viruses by the precipitin test. The viruses³ used were: spot necrosis (Tobacco Virus IV Johnson), ring spot (Tobacco Virus V Johnson), tobacco mosaic (Tobacco Virus I Johnson), and attenuated forms of spot necrosis and tobacco mosaic (5).

EXPERIMENTAL PROCEDURE

It was soon discovered that the various methods used for purification of tobacco mosaic are not adequate for purification of other viruses, so that considerable experimentation on methods of purification was necessary. In the preparation of antigens of spot necrosis, ring spot, and attenuated spot necrosis, precipitation of the virus with safranin and removal of the safranin by adsorption with Lloyd's reagent according to the method described by VINSON (11) proved most satisfactory.

³ Professor JAMES JOHNSON of the Department of Horticulture, University of Wisconsin, kindly supplied the pure strains of the viruses used in this study.

PURIFICATION BY SEITZ FILTER METHOD.—The antigen of Tobacco Virus I was purified and concentrated by adsorption on a Seitz filter and subsequent elution with buffers. Since this method has not been reported previously it is described here in detail. Adsorption of the virus of tobacco mosaic by the Seitz filter, already noted, had suggested the possibility of using it to collect, purify, and concentrate the virus. Accordingly diseased plants were ground in a food chopper, the crushed material frozen and thawed, and the juice pressed out and centrifuged until clear. The supernatant fluid (the crude plant juice) was first passed through a Berkefeld V filter to remove materials not precipitated by centrifuging, and then passed through a Seitz filter. To remove the accumulated plant material from the pad, the Seitz filter was washed with about 200 cc. of distilled water, or until the filtrate was clear and colorless. Experiments on the infectivity of the filtrate show that distilled water does not remove the adsorbed virus from the pad to any appreciable extent.

Filtration is a complex phenomenon and many factors other than pore size are involved. Perhaps the two most important factors determining the filterability of particles are (1) the charge of the particle and of the filter, and (2) the surface tension and the pH of the menstrum. In order to determine which of these factors caused Tobacco Virus I to be adsorbed on the Seitz filter pad, three methods of releasing it were employed: electrophoresis, use of surface tension depressants, and change of pH with buffers.

If the viruses are adsorbed on the Seitz filter because of a difference in the charge of the virus (1) and the filter, it would seem possible to suspend the adsorbed virus in an electrolyte and to release it by electrophoresis. Consequently Seitz filter pads through which 500 cc. of mosaic juice had been passed were ground in 50 cc. of 0.85 per cent NaCl solution. This suspension was placed in a U-tube and electrodes carrying 110 volts of direct current introduced. Samples of the suspension were removed from both poles at 2-, 4-, and 20-hour intervals. The samples from the negative pole contained no virus and those from the positive pole only a small amount, as evidenced by their infectivity when tested by HOLMES'S method. The experiment was repeated on the other viruses with

similar results. It is possible that the virus may have been inactivated or killed by the concentration of chlorine liberated during electrolysis.

Since electrophoresis did not effectively separate the virus from the filter, ethyl alcohol, as a surface tension depressant, was next tried. After filtering 500 cc. of Tobacco Virus I, the Seitz filter was washed alternately with 40 cc. of 95 per cent ethyl alcohol and 10 cc. of water until a total of 100 cc. of liquid had been passed through it. The alcohol was removed from the filtrate by distillation *in vacuo* at 40° C., and the remaining suspension diluted to 500 cc. and tested for infectivity on *Nicotiana glutinosa*. The three plants inoculated with crude untreated juice showed an average of eighteen lesions per leaf, while the three plants inoculated with the alcohol-free filtrate showed an average of forty-five lesions per leaf, thus indicating a relatively high concentration of virus.⁴ The experiment was repeated using methyl alcohol with essentially the same results. The method seems to offer possibilities for the purification and concentration of Tobacco Virus I, which is very resistant to alcohol. However, when the experiment was repeated with spot necrosis, attenuated spot necrosis, and ring spot, the viruses were evidently inactivated or killed by the alcohol.

Finally, elution of the viruses was attempted by the use of SÖRENSEN'S phosphate buffers at pH 6.0, 6.3, 6.6, 7.0, 7.2, 7.6, and 8.2. It seemed probable that since adsorption depends to some extent upon pH, the virus might be released from the filter by the use of buffers. Two hundred cc. of crude mosaic juice was passed through a Seitz filter. The filter was washed with distilled water until the filtrate was clear and colorless, the pads were removed and macerated in 70 cc. of the buffer, and placed in the icebox overnight. This last step was not essential. The pulp was then separated from the virus suspension by filtration through a Gooch asbestos filter. While the results were not always consistent, buffers with a pH of 6.6–7.2 seemed most effective in eluting the virus. Suspensions

⁴ The apparent increase of the virus obtained might be explained on the assumption that the infectious agent exists as aggregates, or is adsorbed on particles that exist as aggregates which are broken up during manipulation.

thus obtained were colorless but slightly opalescent. That they contain a high concentration of virus, as tested by HOLMES'S *Nicotiana glutinosa* method, is indicated by the following protocol.

INOCULUM	AVERAGE NUMBER OF LESIONS ON N. GLUTINOSA
Crude virus.....	16
Virus purified by the Seitz filter method at pH 7 and diluted to 200 cc.....	69
Seitz filtrate.....	4

It will be noted that the Seitz filtrate was slightly infectious. After filtering large amounts of virus, the Seitz filter becomes saturated and some virus passes it, although there seems to be great

TABLE IV

PRECIPITIN TESTS WITH TOBACCO VIRUS I ANTISERA AND PURIFIED
HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

? = INDECISIVE, o = NO REACTION, + = FAINT PRECIPITATE, ++ = SLIGHT BUT
DEFINITE PRECIPITATE, +++ = MODERATE PRECIPITATE,
++++ = HEAVY PRECIPITATE

(READINGS WERE MADE AFTER 8 AND 24 HOURS. INCUBATION, 8 HOURS AT
37° C., 16 HOURS AT ICEBOX TEMPERATURE)

PURIFIED ANTIGENS	ANTISERUM TOBACCO VIRUS I PURIFIED BY SEITZ FILTER METHOD							CONTROLS	
	DILUTIONS							NaCl	NOR- MAL SE- RUM
	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
Tobacco Virus I Seitz filter method.....	++++	++++	++	++	o	o	o	o	o
Spot necrosis Vinson's method.....	o	o	o	o	o	o	o	o	o
Attenuated spot necro- sis Vinson's method.	?	o	o	o	o	o	o	o	o
Healthy plant Vinson's method.....	o	o	o	o	o	o	o	o	o
Ring spot Vinson's method.....	o	o	o	o	o	o	o	o	o

variation in the ease with which this is accomplished. In general the juice from old plants or from plants showing severe symptoms is more effective in saturating the filter than is the juice from young

actively growing plants. Furthermore, if the filter is saturated with paraffin oil, the virus will readily pass it.

This method of purification by adsorption of the virus on the Seitz filter and subsequent elution by the use of buffers, hereafter

TABLE V

PRECIPITIN TESTS WITH SPOT NECROSIS ANTISERUM AND PURIFIED
HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

o=NO REACTION, +=FAINT PRECIPITATE, ++=SLIGHT BUT DEFINITE PRE-
CIPITATE, +++=MODERATE PRECIPITATE, ++++=HEAVY PRECIPITATE

PURIFIED ANTIGENS	ANTISERUM SPOT NECROSIS							CONTROLS	
	DILUTIONS							NaCl	NOR- MAL SE- RUM
	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
Spot necrosis Vinson's method...	++++	++++	++++	++++	++++	++++	++++	+	o
Attenuated spot nec- rosis Vin- son's method...	++	++	++	+	+	+	+	o	o
Healthy plant....	o	o	o	o	o	o	o	o	o
Tobacco Vi- rus I Seitz filter method...	o	o	o	o	o	o	o	o	o
Ring spot Vinson's method...	++	++	++	++	o	o	o	o	o

designated as the Seitz filter method, was used in preparing Tobacco Virus I; and in a later experiment, in purifying cucumber mosaic. It has several advantages: it is simple and rapid, and does not involve the use of chemicals harmful to the virus.

PRODUCTION OF ANTISERA.—The antigens of Tobacco Virus I, purified according to the Seitz method; of spot necrosis, attenuated spot necrosis, and ring spot, purified according to VINSON'S method; and of a healthy plant were given to rabbits in a series of eleven injections of 3 cc. each. On the sixth day after the last injection the rabbits were exsanguinated, the blood allowed to clot, and the sera collected.

PRECIPITIN TESTS.—Precipitin ring tests were set up with homologous and heterologous antigens. The results were not satisfactory as the titres were too low. This was attributed to the fact that the antigens were not concentrated enough to allow dilution.

Regular precipitin tests were then run. In this case the dilutions of antigen were held constant and those of the antisera varied. The

TABLE VI

PRECIPITIN TESTS WITH ATTENUATED SPOT NECROSIS ANTISERUM AND
PURIFIED HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

o=NO REACTION, +=FAINT PRECIPITATE, ++=SMALL BUT DEFINITE PRECIPITATE, +++=MODERATE PRECIPITATE, ++++=HEAVY PRECIPITATE

PURIFIED ANTIGENS	ANTISERUM ATTENUATED SPOT NECROSIS							CONTROLS	
	DILUTIONS							NaCl	NOR- MAL SE- RUM
	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
Attenuated spotnecrosis Vinson's method....	++++	++++	++++	++++	+++	++	++	o	o
Spot necrosis Vinson's method....	++++	++++	++++	+++	++	+	+	o	o
Ring spot Vin- son's meth- od.....	++	++	++	++	o	o	o	o	o
Tobacco Virus I Seitz filter method....	o	o	o	o	o	o	o	o	o
Healthy plant	o	o	o	o	o	o	o	o	o

sera were diluted with 0.85 per cent NaCl. Each precipitin tube contained 0.5 cc. of serum and 0.5 cc. of antigen.

Table IV shows that antisera for Tobacco Virus I, purified according to the Seitz method, reacts with its homologous antigen but not with antigens of the other viruses nor with the antigen of a healthy plant. This indicates that there is no serological relationship between Tobacco Virus I and the other viruses, and that the normal plant antigen had been removed by the purification methods employed.

Tables V, VI, and VII show (1) that the purified preparations of

spot necrosis, attenuated spot necrosis, and ring spot are antigenic; (2) that these viruses are not related serologically to Tobacco Virus I; and (3) that there appears to be a serological relationship between the viruses of ring spot and of spot necrosis. No significant serological difference is evidenced between spot necrosis and attenuated spot necrosis.

TABLE VII

PRECIPITIN TESTS WITH HEALTHY TOBACCO ANTISERUM AND HOMOLOGOUS AND PURIFIED HETEROLOGOUS ANTIGENS

PURIFIED ANTIGENS	ANTISERUM HEALTHY TOBACCO PLANT							CONTROLS	
	DILUTIONS							NaCl	NOR- MAL SE- RUM
	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
Healthy plant.	++++	++++	++++	++++	++	++	?	o	o
Tobacco Virus I Seitz filter method. . . .	o	o	o	o	o	o	o	o	o
Spot necrosis Vinson's method. . . .	++	++	o	o	o	o	o	o	o
Attenuated spot necrosis Vinson's method. . . .	++	++	o	o	o	o	o	o	o
Ring spot Vin- son's method	o	o	o	o	o	o	o	o	o

PRECIPITIN ABSORPTION TESTS

In order to check further the results of precipitin tests with purified antigens, precipitin absorption tests were run on the antisera prepared against the following antigens:

1. Tobacco mosaic Virus I, purified by the Seitz filter method.
2. Tobacco mosaic Virus I, crude.
3. Attenuated tobacco mosaic, Virus I, attenuated by growing plants at a high temperature.
4. Spot necrosis.
5. Ring spot.⁵
6. Cucumber mosaic, purified by the Seitz method.

⁵ This strain was obtained from Dr. R. G. HENDERSON of the Department of Plant Pathology, Virginia Agricultural Experiment Station, Blacksburg.

7. Crude cucumber juice, cleared by supercentrifuging at 33,000 r.p.m.
8. Healthy tobacco plant, cleared by passing through Berkefeld V filters.

TABLE VIII

SUMMARY OF PRECIPITIN TESTS WITH ABSORBED SERA IN THE PRESENCE OF
HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

o=NO PRECIPITATE, +=SLIGHT PRECIPITATE, ++=DEFINITE PRECIPITATE,
+++ = MODERATE PRECIPITATE, ++++ = HEAVY PRECIPITATE

ANTIGENS (DILUTION 1:3)	TUBES	ANTISERA (DILUTION 1:10)							
		TOBACCO VIRUS I, SEITZ FILTER METHOD	VIRUS I, CRUDE	AT- TENU- ATED MOSAIC	SPOT NECRO- SIS	RING SPOT	CU- CUMBER MOSAIC, FILTER METHOD	CU- CUMBER MOSAIC, CRUDE	HEAL- THY TO- BACCO PLANT
Tobacco Virus I, Seitz filter method.....	{ 1	++++	++++	+++	o	o	o	o	o
	{ 2	++++	++++	+++	o	o	o	o	o
Tobacco Virus I, crude.....	{ 1	++	++	++	o	o	o	o	o
	{ 2	+++	++	++	o	o	o	o	o
Attenuated mo- saic.....	{ 1	+++	++	+++	o	o	o	o	o
	{ 2	++	++	+++	o	o	o	o	o
Spot necrosis..	{ 1	o	o	o	+	o	o	o	o
	{ 2	o	o	o	+	o	o	o	o
Ring spot.....	{ 1	o	o	o	o	o	o	o	o
	{ 2	o	o	o	o	o	o	o	o
Cucumber mo- saic, Seitz fil- ter method..	{ 1	o	o	o	o	o	+	++	o
	{ 2	o	o	o	o	o	+	++	o
Cucumber mo- saic, crude...	{ 1	o	o	o	o	o	+	+	o
	{ 2	o	o	o	o	o	+	+	o
Healthy tobac- co plant.....	{ 1	o	o	o	o	o	o	o	+++
	{ 2	o	o	o	o	o	o	o	+++

Rabbits were immunized as previously described to each of these antigens. The antisera thus produced were absorbed with healthy plant antigen as follows. One and five-tenths cc. of a healthy plant was added to 3 cc. of each antiserum, the mixture incubated at 37° C. for four hours, placed in an icebox overnight, and centrifuged. The

supernatant liquid was pipetted into sterile test tubes and 1.5 cc. of healthy plant antigen was again added. This process of absorption was repeated until no precipitate formed upon the further addition of healthy plant antigen.

PRECIPITIN TESTS

The absorbed antisera were then tested for precipitins with homologous and heterologous antigens. Five-tenths of a cubic centimeter of 1:10 dilution of antiserum was added to 0.5 cc. of 1:3 dilution of antigen. These dilutions of antigen and antiserum were employed because preliminary experiments had shown that if cross-reactions occurred, they were most likely to appear under these conditions.

The results of these tests, summarized in table VIII, indicate that when precipitins of a healthy plant have been removed from the antiserum by absorption with healthy plant antigen, the serum gives a positive test only with the homologous virus antigen. This confirms the results of tests with purified antigens. No difference could be detected between Tobacco Virus I and an attenuated form. The absorbed ring spot antiserum did not give any reaction with homologous or heterologous antigen. The titre of the ring spot antisera with homologous antigen was 1:8, consequently the dilution that resulted when the serum was absorbed was too great to give a reaction.

Summary

1. The juice from virus-diseased plants contains, in addition to the antigenic constituents of a normal healthy plant, an antigenic fraction which, by the methods employed, is inseparable from the virus itself. This holds true in the case of Tobacco Virus I whether the virus is grown in tobacco or in tomato. This antigenic factor not only accompanies the virus but is specific for a particular virus, in that the antibodies induced by one virus are qualitatively different from those induced by other viruses.

2. Viruses may be freed from the antigenic constituents of healthy plants by several methods of purification. A new method by use of a Seitz filter is described. Regardless of the method of purification, however, it was not possible to separate the virus, as judged by infectivity, from the specific antigenic factor which accompanies it.

3. Close association of the antigenic factor with infectivity and

the specific nature of the antigenic fractions accompanying the different viruses strongly suggest that this specific antigenic factor is either the virus itself or a virus-plant-protein complex in which the virus plays the rôle of a haptene. Whatever the true explanation of the nature of the specificity of the reaction may be, the reaction seems to be specific for the virus. The precipitin test should prove to be a valuable aid in the further classification of plant viruses.

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